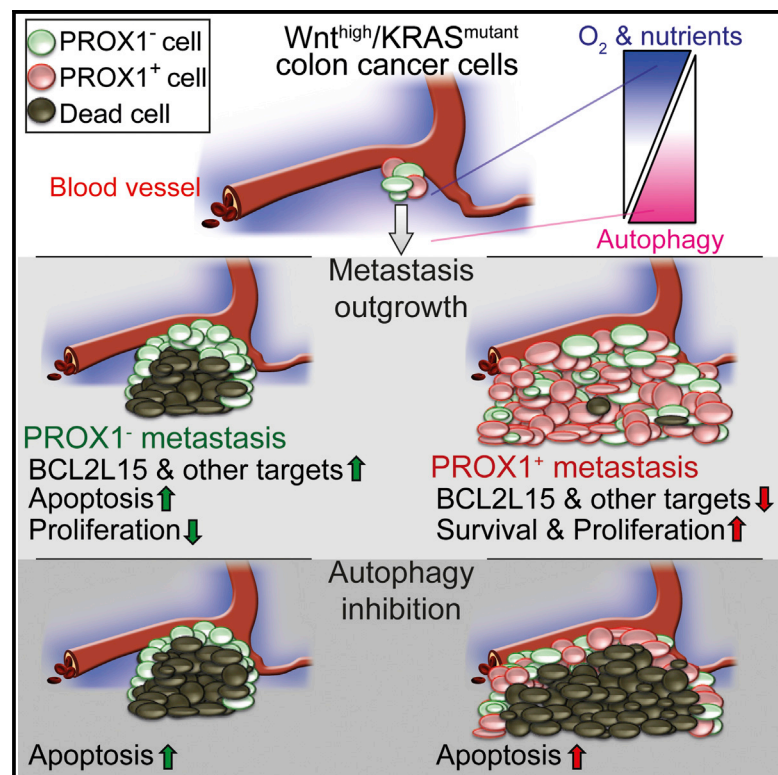


Cell Reports

PROX1 Promotes Metabolic Adaptation and Fuels Outgrowth of Wnt^{high} Metastatic Colon Cancer Cells

Graphical Abstract



Highlights

PROX1 is expressed in stem-like/progenitor cell subpopulation in Wnt^{high} metastases

PROX1 promotes autophagy-dependent metabolic adaptation and apoptosis evasion

Genome-wide analyses identify direct PROX1 targets, such as pro-apoptotic BCL2L15

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In Brief

Abnormal Wnt pathway activation is involved in the initiation of most colorectal cancers; however, little is known about its contribution to metastatic outgrowth. Ragusa et al. report that the Wnt target transcription factor PROX1 is produced in a subpopulation of colon cancer stem/progenitor cells and orchestrates the transcriptional network responsible for autophagy-dependent survival of metastases. Inactivation of PROX1 or PROX1-associated pathways prevents expansion of macrometastases and may provide new targets for therapeutic intervention in Wnt^{high} colorectal cancer.

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PROX1 Promotes Metabolic Adaptation and Fuels Outgrowth of Wnt^{high} Metastatic Colon Cancer Cells

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SUMMARY

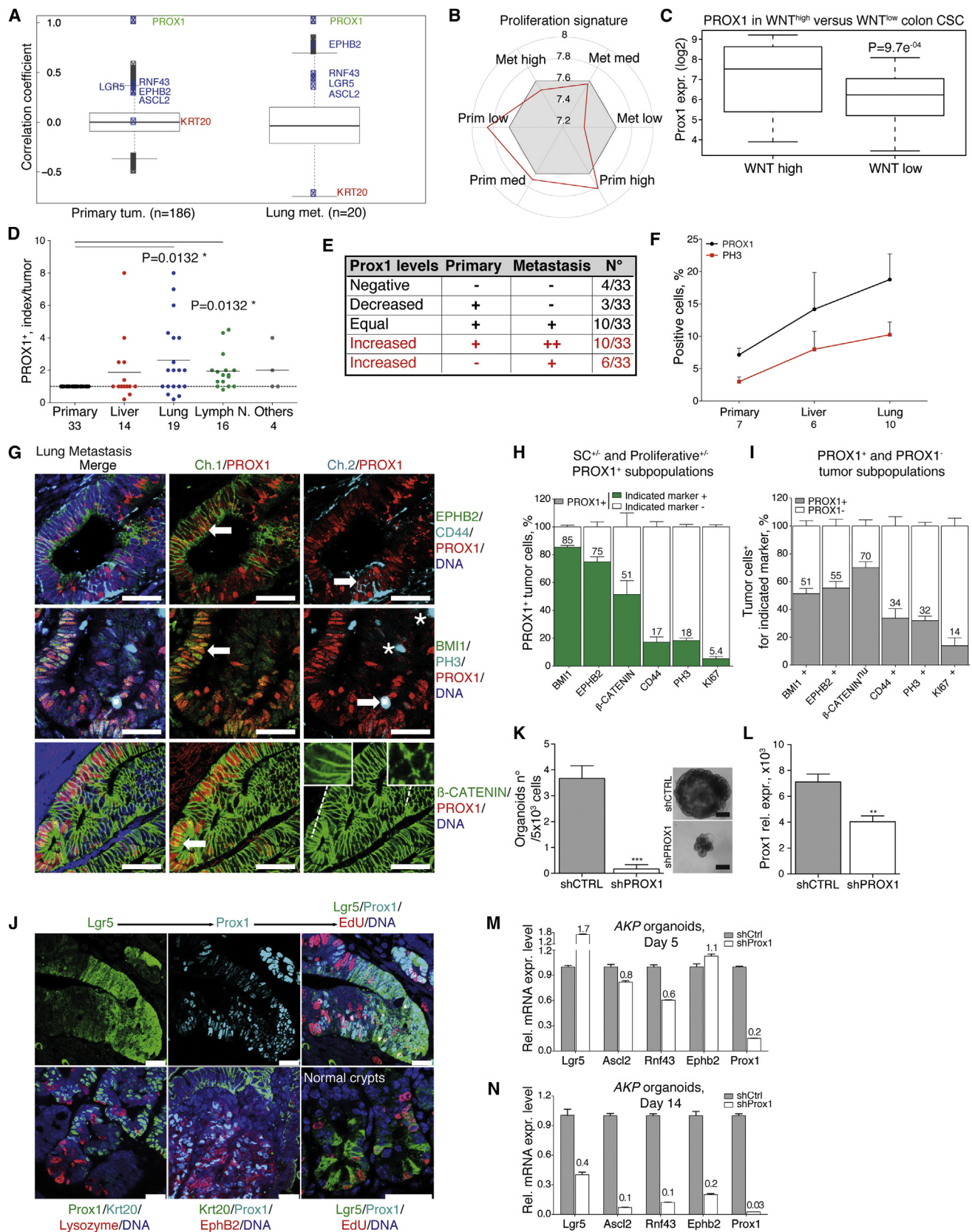
The Wnt pathway is abnormally activated in the majority of colorectal cancers, and significant knowledge has been gained in understanding its role in tumor initiation. However, the mechanisms of metastatic outgrowth in colorectal cancer remain a major challenge. We report that autophagy-dependent metabolic adaptation and survival of metastatic colorectal cancer cells is regulated by the target of oncogenic Wnt signaling, homeobox transcription factor PROX1, expressed by a subpopulation of colon cancer progenitor/stem cells. We identify direct PROX1 target genes and show that repression of a pro-apoptotic member of the BCL2 family, BCL2L15, is important for survival of PROX1⁺ cells under metabolic stress. PROX1 inactivation after the establishment of metastases prevented further growth of lesions. Furthermore, autophagy inhibition efficiently targeted metastatic PROX1⁺ cells, suggesting a potential therapeutic approach. These data identify PROX1 as a key regulator of the transcriptional network contributing to metastases outgrowth in colorectal cancer.

INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer-related mortality. The prognosis is especially poor for metastatic CRC, with overall 5-year survival rates of 5%–8% (Cunningham

et al., 2010). Current treatment options include resection of solitary metastases and combinations of anti-vascular endothelial growth factor (VEGF) or epidermal growth factor receptor (EGFR) inhibitors and cytotoxic therapy. However, these targeted therapies are effective only in a small subset of patients with metastatic disease and treatment benefits are short-lived, further highlighting the need for a better understanding of metastatic CRC (De Roock et al., 2011).

Wnt signaling is essential for the maintenance of normal intestinal stem cells and is aberrantly activated in a significant proportion of CRCs (Clevers, 2013). The evolutionary conserved transcription factor PROX1 is a Wnt target gene in colon adenomas, where it contributes to the transition from early to dysplastic stage (Petrova et al., 2008). Prox1 is not expressed in normal intestinal stem cells, demonstrating that only oncogenic Wnt signaling triggers Prox1 expression (Petrova et al., 2008). Increased epithelial-to-mesenchymal transition (EMT) was recently reported in PROX1-expressing CRC cells in vitro (Lu et al., 2012). However, the role of PROX1 in metastatic CRC has not been investigated. Here, we report that PROX1 is expressed in stem-like cells in a subset of Wnt^{high} colon carcinomas and metastases. PROX1 acts as a key regulator of macrometastatic lesions outgrowth by promoting adaptation of tumor cells to hypoxia and nutrient deprivation. Furthermore, we show that targeting autophagy efficiently eliminates the growth advantage of PROX1⁺ cells. We identify direct PROX1 target genes in CRC cells and show that a pro-apoptotic member of BCL2 family, BCL2L15, is one of the downstream effectors of PROX1. Our data thus identify a cancer-specific component of the Wnt signaling required for outgrowth of metastases and suggest that inhibition of PROX1, or PROX1-regulated pathways, might constitute a new therapeutic approach.



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RESULTS

PROX1 Is Expressed in Progenitor/Stem Cells in Wnt^{high} Colon Cancer Metastases

We examined whether PROX1 expression correlates with Wnt signaling, stem-like/progenitor markers, cell differentiation, and proliferation, using transcriptomes of 166 primary CRCs and 20 lung metastases (Sheffer et al., 2009). PROX1 expression significantly correlated with stem/progenitor cell markers and Wnt targets LGR5, ASCL2, RNF43, and EPHB2 (Clevers, 2013) in primary tumors, and this correlation was further significantly increased in lung metastases (Figure 1A; $p < 0.05$). In contrast, PROX1 expression in metastases anticorrelated with the differentiation marker KRT20 (Figure 1A). Analysis of the proliferation signature MKI67, FOXM1, MYC, and AURKB (Jung et al., 2011) revealed that PROX1 levels did not correlate with the signature in primary tumors, whereas PROX1^{low} metastases had a lower proliferation index (Figure 1B).

We next asked how PROX1 expression correlates with molecular subtypes of CRC described by Budinska et al. (2013) and Sadanandam et al. (2013). Highest PROX1 levels were found in Wnt^{high} subtypes in both classification schemes (Figures S1A and S1B). In contrast, PROX1 was low in more-differentiated subtypes, such as the subtype A with high expression of differentiation markers and subtype C with high frequency of cancer-specific DNA hypermethylation (CIMP-H) of the Budinska et al. classification. Most of CIMP CRCs are also mismatch repair (MMR) deficient due to hypermethylation of the *MLH1* gene promoter (Truninger et al., 2005). In agreement with these results, PROX1 was low in MMR-deficient CRCs (Figures S1C–S1E). We further confirmed these data using the Pan-European Trials in Alimentary Tract Cancers database, containing microsatellite instability status analysis for 688 CRC primary colon carcinomas (Roth et al., 2010; Figure S1F).

The bioinformatics analysis predicted that PROX1 correlates with stem/progenitor markers, high Wnt signaling, and prolifera-

tion and anticorrelates with differentiation markers. Interrogation of transcriptomes from human CRC stem cells, stratified by the levels of Wnt signaling (de Sousa E Melo et al., 2011), demonstrated that PROX1 was significantly higher in Wnt^{high} versus Wnt^{low} cancer stem cells (Figure 1C; $p = 9.7 \times 10^{-4}$). To evaluate PROX1 protein levels in metastasis versus primary tumor, we stained 33 matched primary CRCs and their metastases. Lung and lymph node metastases had significantly higher levels of PROX1 in comparison to the primary tumors, with a positive trend for other metastatic sites (Figures 1D, 1E, and S1G). To establish whether PROX1 is expressed in stem/progenitor cells, we analyzed coexpression of PROX1 and EPHB2, CD44 and BMI1, previously shown to encompass progenitor/stem cell populations (Todaro et al., 2014; Martorell et al., 2014; Merlos-Suárez et al., 2011; Kreso et al., 2014). We also analyzed β -catenin nuclear/cytoplasmic localization as a marker of Wnt pathway activation, proliferation, and differentiation. In agreement with the bioinformatics analyses, differentiated KRT20⁺ cells did not express PROX1 and, in metastases, cell proliferation correlated with levels of PROX1 (Figures 1F and S1H–S1J). The majority of PROX1⁺ cells also coexpressed EPHB2 and BMI1, 50% had nuclear/cytoplasmic β -catenin, and 17% coexpressed CD44 or proliferation markers. A significant proportion of EPHB2⁺, BMI1⁺, CD44⁺, and PH3⁺ cells did not express PROX1 (Figures 1G–1I). Thus, in human metastases, PROX1 partially colocalizes with markers of stem/progenitor cells, proliferation, and activated WNT signaling.

To extend our observations to a model with a well-characterized stem cell compartment, we analyzed adenomas from *Apc^{fl/fl};Lgr5-EGFP-Ires-CreERT2* mice. GFP marks *Lgr5*⁺ normal and transformed stem cells, which give rise to differentiated progeny in both normal intestine and adenoma (Schepers et al., 2012). Fifty to sixty percent of GFP^{high}, GFP^{low}, EphB2⁺, or proliferating 5-ethynyl-2'-deoxyuridine (EdU)⁺ cells expressed Prox1, whereas Prox1 was absent in differentiated lysozyme⁺ Paneth cells or Krt20⁺ enterocytes (Figures 1J and S1K). In line

Figure 1. PROX1 Is Expressed in Stem-like Cells in Wnt^{high} CRC Metastases

- (A) PROX1 expression in CRC metastases correlates with activated Wnt pathway and stem-like/progenitor signature, and it anticorrelates with differentiation marker KRT20. Correlation analysis is based on GSE41258 (Sheffer et al., 2009). Data are presented as box and whiskers; Spearman correlation coefficient: $1.5 \times$ interquartile range (IQR).
- (B) PROX1 expression in CRC metastases correlates with proliferation. Samples from primary tumors and lung metastases (Sheffer et al., 2009) were divided in three categories by PROX1-expression levels. The average expression of proliferation signature (black line) is plotted in log₂ across the six categories. The gray area represents the average expression of all genes.
- (C) WNT^{high} CRC stem cells produce higher levels of PROX1. Expression analysis is based on GSE33112 (de Sousa E Melo et al., 2011). Data are presented as box and whiskers; Log expression values: $1.5 \times$ IQR.
- (D) PROX1 protein is increased in CRC metastases. Samples were scored as shown in Figure S1C. Student's t test with FDR correction; * $p < 0.05$.
- (E) Summary of PROX1 expression analyses in 33 primary tumors and metastases.
- (F) PROX1 protein levels correlate with proliferation in CRC metastases. Percentages of PROX1⁺ and phosphohistone H3⁺ cells in primary tumors ($n = 7$) and corresponding lung ($n = 10$) and liver ($n = 6$) metastases. Data are presented as mean \pm SEM.
- (G) PROX1 is expressed in a subset of progenitor/stem cells in CRC metastases. Staining for EPHB2, CD44, BMI1, β -catenin, and phosphohistone H3. Inserts: examples of cytoplasmic and membranous localization of β -catenin. White arrows: cells coexpressing PROX1 and the indicated marker. The scale bar represents 50 μ m.
- (H and I) Quantification of PROX1 expression data shown in (G). PROX1 expression in the indicated human tumor cell populations (H) or marker expression levels in PROX1⁺ tumor cells (I). Data are presented as mean \pm SEM.
- (J) Prox1 is expressed in a subset of mouse transformed intestinal stem/progenitor cells. Staining for Prox1, GFP, EphB2, Krt20, or lysozyme in *Apc^{fl/fl};Lgr5-EGFP-Ires-CreERT2* adenomas or normal intestine. Proliferating cells were identified by EdU incorporation assay. The scale bar represents 20 μ m.
- (K) Prox1 depletion decreases clonogenic growth of AKP organoids. AKP cells were transduced with the control or PROX1 shRNAs, sorted to isolate single GFP⁺ cells, and plated in Matrigel. Colony number/well was determined at day 15. Student's t test; *** $p < 0.001$. Data are presented as mean \pm SEM.
- (L) Prox1 mRNA levels in the control or shPROX1 transduced AKP organoids. Student's t test; ** $p < 0.01$. Data are presented as mean \pm SEM.
- (M and N) Stem/progenitor and Wnt target gene expression in AKP organoids following Prox1 depletion at days 5 and 14. Data are presented as mean \pm SEM.

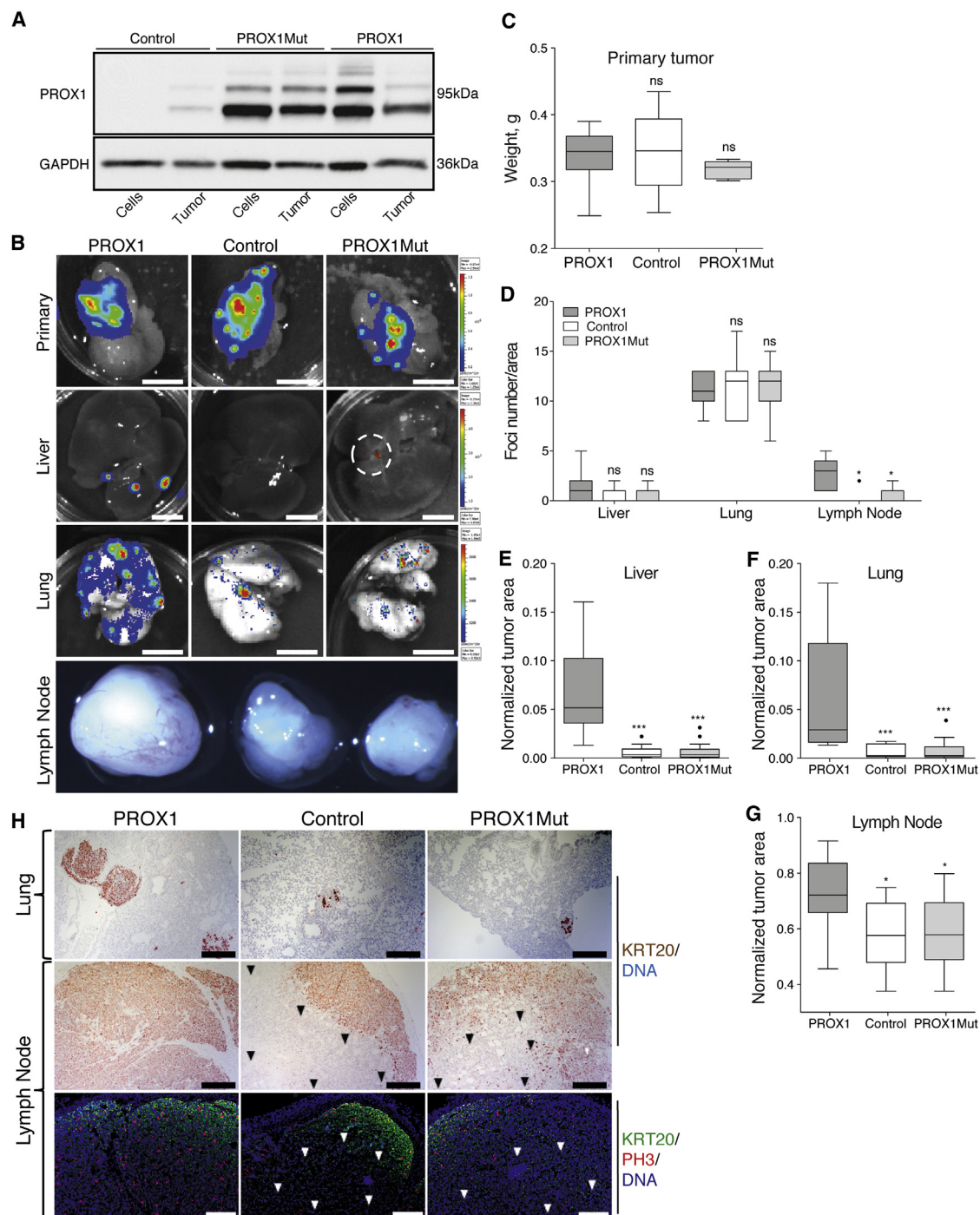


Figure 2. PROX1 Controls CRC Metastasis

(A) PROX1 levels in DLD1-PROX1, DLD1-PROX1mut, or control cells and tumor lysates. Western blotting for the indicated proteins.

(B) PROX1 promotes growth of metastases. Luciferase-tagged DLD1 cells overexpressing wild-type PROX1 (PROX1), mutated PROX1 (PROX1Mut), or transfected with control vector (control) were implanted intracably in nonobese diabetic (NOD)-severe combined immunodeficiency (SCID); *IL-2R γ ^{-/-}* mice and detected by bioluminescent imaging. The scale bar represents 8 mm.

(C) Comparable size of primary tumors. $n = 9/\text{group}$. Data are presented as box and whiskers; Tukey appearance.

(D) PROX1 overexpression does not affect metastasis incidence. Tumor cells were identified by staining for KRT20. Data are presented as box and whiskers; Tukey appearance.

(E–G) PROX1 promotes growth of individual metastases. One-way ANOVA; * $p < 0.05$; *** $p < 0.001$. Data are presented as box and whiskers; Tukey appearance.

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with previous results, normal intestinal stem cells were Prox1⁺ (Petrova et al., 2008; Figures 1J and S1L). These data show that, as in human tissues, a subset of transformed intestinal stem cells produces high levels of Prox1. Furthermore, a proportion of GFP^{low}/EphB2⁺/EdU⁺ adenoma progenitor cell population, but not more-differentiated cells, maintain Prox1 expression. We conclude that (1) PROX1 expression partially overlaps with other cancer stem cell (CSC)/progenitor markers, (2) many proliferating cells in the tumors are PROX1-negative, (3) PROX1 is not expressed in differentiated cells, and (4) PROX1 expression is increased in metastases in comparison to the primary tumors. Thus, PROX1 is induced only upon transformation and it defines a subset of cancer stem/progenitor cells, which themselves are moderately proliferative but, when present in metastases, signify high proliferative activity.

PROX1 Controls CSC Colony-Forming Ability

To study whether PROX1 contributes to the clonogenic growth of CRC cells, we used intestinal organoids from *Apc*^{fl/fl}; *Kras*^{Lsl-G12D}; *Tp53*^{fl/fl}; *villin-CreERT2* (AKP) mice. In addition to high Wnt signaling, such intestinal epithelial cells carry an oncogenic mutation in *Kras* and loss of the tumor suppressor p53, thus recapitulating major pathways important for development of CRC (Fearon, 2011). Similar to human CRCs and *Apc*^{fl/fl}; *Lgr5-EGFP-Ires-CreERT2* adenomas, we observed partially overlapping localization of Prox1 and intestinal stem cell/progenitor markers Lgr5, nuclear β -catenin, EphB2, and Cd44; proliferation markers phosphohistone H3 and Ki67; and absence of Prox1 expression in differentiated Krt20⁺ and Krt6⁺ cells (Figures S1M and S1N). We next tested whether Prox1 is necessary to sustain growth of AKP organoids in a colony-forming assay, where a single stem cell gives rise to a 3D “organoid” structure (Sato et al., 2011). Downregulation of Prox1 significantly decreased the number of colonies formed by single AKP cells, thus demonstrating that Prox1 maintains clonogenic growth (Figures 1K and 1L). Expression of progenitor/stem cell markers and Wnt target genes Lgr5, Ascl2, Rnf43, and EphB2 was not modified in early organoids at day 5, despite downregulation of Prox1 (Figure 1M). However, their expression was lower in organoids by day 15 (Figure 1N). Taken together with our previous data showing that Prox1 does not affect general Wnt reporter activity (Petrova et al., 2008), these results indicate that Prox1 does not directly regulate stem/progenitor gene expression or general Wnt signaling, but it rather maintains the clonogenic capacity of CSCs.

PROX1 Controls CRC Metastasis

Analyses of human tumor samples indicated that PROX1 expression is elevated in a subset of metastases (Figure 1E), consistent with potential involvement of PROX1 in metastatic CRC. To clarify this, we used an orthotopic model, in which CRC cells form metastases in lymph nodes, liver, and lungs after intracecal injection (Céspedes et al., 2007). To study if PROX1 is

sufficient to promote metastasis, we overexpressed PROX1 and its inactive form PROX1mut, bearing mutations in the DNA-binding domain, in PROX1-negative DLD1 CRC cells. As another control, we used cells transfected with an empty vector (Figure 2A). The primary tumor size was similar between the groups, and there was no difference in primary tumor weights (Figures 2B and 2C). However, significantly more DLD1-PROX1 cells were present in lungs, liver, and lymph nodes (Figure 2B). Metastatic incidence was increased only in lymph nodes of PROX1 group (Figure 2D). In all tissues, PROX1⁺ metastases were 2- to 5-fold larger than the control lesions (Figures 2E–2G). PROX1⁺ metastases displayed higher levels of proliferation marker phosphohistone H3. Strikingly, proliferating cells were observed throughout the lesion in DLD1-PROX1 metastases, whereas in the control, metastases proliferation was restricted to the outer rim as a result of massive central necrosis (Figure 2H).

We next asked whether PROX1 depletion in PROX1⁺ SW620 cells affects development of metastases (Figures S2A–S2C). Depletion of PROX1 did not affect growth of primary tumors; however, we observed marked differences in the development of metastases (Figures S2D–S2F). In this case, there were more PROX1⁺ metastases, which were also bigger in comparison to the controls (Figures S2E and S2F). Depletion of PROX1 reduced metastases proliferation and induced extensive necrosis and apoptosis, whereas little cell death was observed in primary tumors (Figures S2G and S2H).

To confirm that these phenotypes were not the result of long-term cell selection, we studied SW480R/PROX1si cells, in which PROX1 is suppressed upon administration of doxycycline (Petrova et al., 2008; Figure S2I). Depletion of PROX1 reduced metastases size and incidence without affecting the primary tumor (Figures S2J and S2K). Doxycycline treatment had no effect on SW480R/GFPsi metastases, in which a control small hairpin RNA (shRNA) was induced (Figures S2J and S2L). Similar to other models, suppression of PROX1 resulted in formation of large central necrotic areas (Figure S2M). In summary, loss- and gain-of-function experiments show that PROX1 is necessary and sufficient to promote CRC metastases.

PROX1 Fuels Metastases Outgrowth

Metastasis requires several steps: invasion, extravasation, survival in the circulation, colonization of distal organs, and outgrowth of macrometastases (Chaffer and Weinberg, 2011). PROX1 promoted EMT in vitro by suppressing E-cadherin (Lu et al., 2012). However, in our hands, PROX1⁺ and PROX1⁺ cells expressed comparable levels of E-cadherin and EMT marker vimentin (Figures 3A and S3A). PROX1 did not increase invasion in vitro assays or local invasion in primary tumors, and vascular density was comparable in PROX1⁺ versus PROX1⁺ tumors (Figures S3B–S3E). Thus, enhanced local invasion and angiogenesis are not major contributing factors in our models.

To test the role of PROX1 during the subsequent steps of metastasis, we injected enhanced GFP (EGFP)-tagged SW620

(H) Only wild-type PROX1 promotes growth of metastases. Upper row: increased size of DLD1-PROX1 lung metastases. Middle row: decreased cell death in DLD1-PROX1 metastases. Arrows, necrotic areas. Lower row: increased proliferation in DLD1-PROX1 metastases. Chromogenic staining for KRT20 (brown) or fluorescent staining for phosphohistone H3 (red), KRT20 (green), and DNA (blue). The scale bars represent 200 μ m (upper and middle panels) and 100 μ m (lower panel).

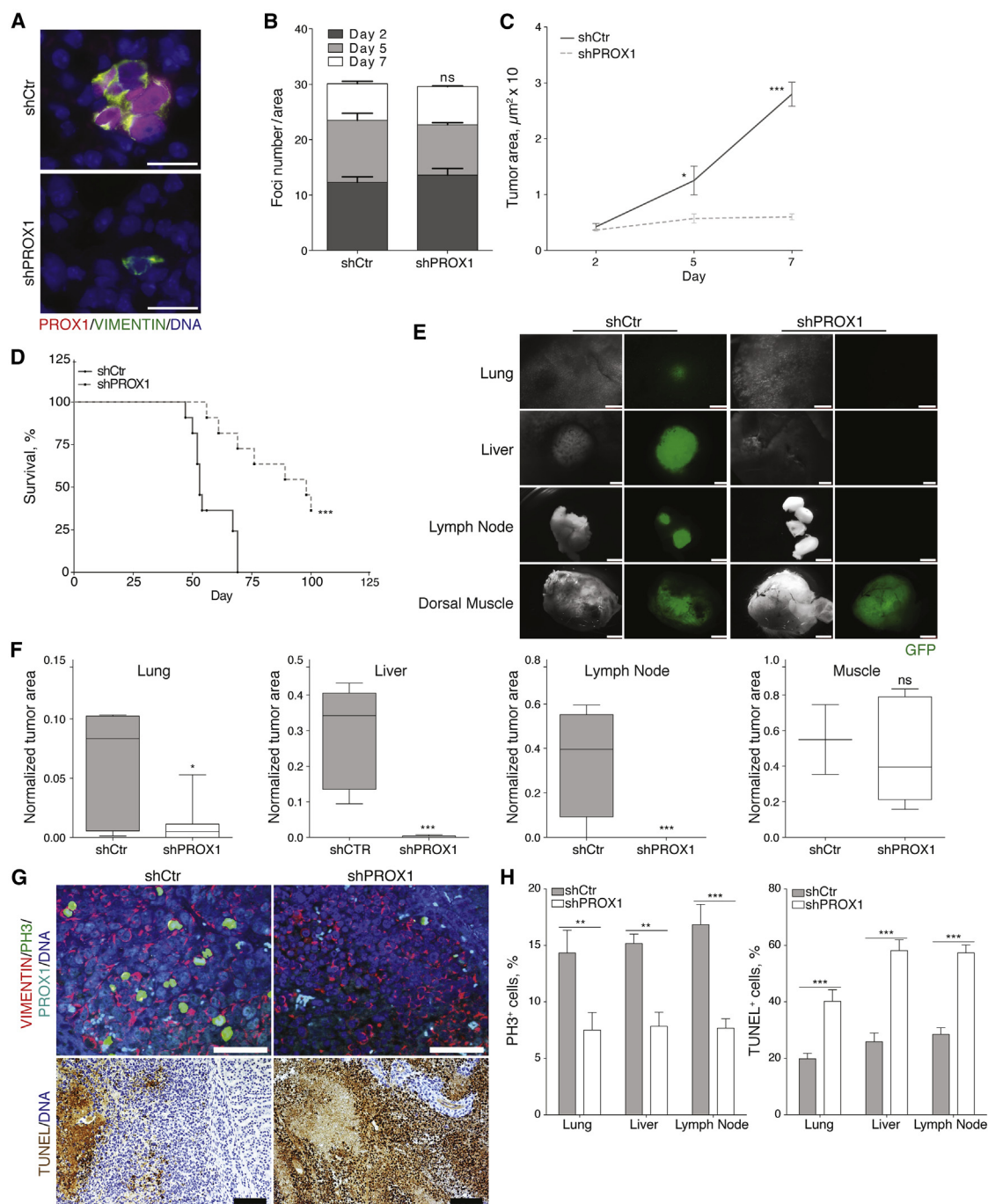


Figure 3. PROX1 Promotes Metastatic Outgrowth

(A) Lung micrometastases 3 days after the tail vein injection of SW620-shCtrl or SW620-shPROX1 cells. Staining for PROX1 (red), vimentin (green), and DNA (blue). The scale bar represents 20 μm .

(B) PROX1 is dispensable for tumor cell extravasation. Number of lung metastatic foci after injection of SW620-shCtrl or SW620-shPROX1 cells into the tail vein of NOD-SCID; *IL-2R γ ^{-/-}* mice. Tumor cells were stained for human vimentin. Data are presented as mean \pm SEM.

(C) PROX1 promotes growth of metastases. Average size was calculated by dividing total metastases area by number of metastases and normalized to tissue area analyzed. Two-way ANOVA; * $p < 0.05$; *** $p < 0.001$. Data are presented as mean \pm SEM.

(D) PROX1 depletion prolongs survival. Kaplan-Meier survival curve of mice injected with SW620-shCtrl or SW620-shPROX1 cells. Log rank (Mantel-Cox) test; *** $p < 0.001$.

(E) PROX1 depletion prevents growth of macrometastases. Ex vivo analysis of SW620-shCtrl and SW620shPROX1 EGFP⁺ cells 7 weeks after tail vein injection into NOD-SCID; *IL-2R γ ^{-/-}* mice. The scale bars represent 750 μm (lung), 1 mm (liver), and 2.5 mm (lymph node and dorsal muscle).

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PROX1⁺ and PROX1[−] cells into the tail vein. PROX1⁺ or PROX1[−] CRC cells formed similar number of lung colonies at days 2, 5, and 7 post-injection (Figures 3A and 3B). Thus, PROX1 is dispensable for tumor cell survival in the blood stream or in early metastases. However, PROX1 depletion prevented further metastatic outgrowth and increased animal survival (Figures 3C and 3D). PROX1[−] cells were unable to establish colonies in most organs, with a notable exception of skeletal muscle (Figures 3E and 3F). Similar to orthotopic models, proliferation and apoptosis were increased and decreased, respectively, in PROX1⁺ metastases compared to PROX1[−] lesions, as determined by phosphohistone H3 staining and TUNEL assay (Figures 3G and 3H). Overall, these results indicate that PROX1 mainly promotes outgrowth of tumor cells in target organs.

PROX1 Promotes Resistance to Hypoxia and Nutrient Deprivation

To investigate the underlying mechanism of PROX1-mediated metastatic outgrowth, we analyzed cell growth under standard cell culture conditions. Modulation of PROX1 levels in three human CRC lines did not affect cell growth in 2D (Figures 4A and S4A). We next studied the ability of these cells to form tumor spheroids. In agreement with AKP organoid results, in all three lines, PROX1⁺ spheroid size and number were increased in comparison to PROX1-negative spheroids (Figures 4B–4E and S4B–S4D). Consistent with in vivo models, PROX1⁺ spheroids proliferated more and underwent apoptosis less than PROX1[−] spheroids (Figures 4F–4I and S4D).

In tumor spheroids, diffusion of nutrients and oxygen is decreased in the center, resulting in a hypoxic/low-nutrient environment and cell death (Hirschhaeuser et al., 2010). Whereas the central part of PROX1[−] spheroids was filled with dead cells, PROX1⁺ spheroids cores contained live cells and more cells proliferated at a distance from the surface (Figure 4J), suggesting that PROX1 promotes cell survival and proliferation in hypoxia and nutrient-poor environments. To directly test this, we cultured DLD1-PROX1 and DLD1 control cells in 0.1% O₂ and/or under reduced glucose or glutamine conditions. Hypoxia induced stabilization of HIF1 α in both PROX1[−] and PROX1⁺ DLD1 cells (Figures 5A and 5B). However, by 72 hr, DLD1 control cells became growth arrested and apoptotic, as determined by staining for activated caspase-3 (Figures 5C–5F). In contrast, hypoxic DLD1-PROX1 cells had somewhat enhanced proliferation and low cell death (Figures 5D, 5F, and 5G). PROX1⁺ cells were also more resistant to cell growth arrest and cell death elicited by the low glucose or glutamine, but not by etoposide, which induces apoptosis through DNA damage (Figures 5D, 5F, 5G, and S5A). Similar results were observed in another cell line SW620 (Figures S5A–S5D). These data indicate that PROX1 sustains cell survival and proliferation under metabolic stress.

PROX1 Promotes Metabolic Adaptation

Increased glycolysis is one of the key characteristics of cancer cells. However, cancer cells can also use oxidative phosphorylation rather than glycolysis to sustain growth (e.g., Haq et al., 2013; Janiszewska et al., 2012). To better understand the mechanisms of increased fitness of PROX1⁺ CRC cells, we studied their metabolic responses upon nutrients or oxygen deprivation. Extracellular acidification rate (ECAR), a measure of glycolysis, and cellular oxygen consumption rate (OCR), a measure of oxidative phosphorylation, were comparable between DLD1-PROX1⁺ and PROX1[−] cells under basal cell culture conditions (Figures 5H and 5I). As expected, glucose deprivation decreased glycolysis in both cells types (Figure 5H). PROX1[−] cells displayed little compensatory increase in OCR, indicative of their inability to switch to an alternative fuel source. In contrast, OCR was high in PROX1⁺ cells upon glucose deprivation, which also paralleled increased survival of PROX1⁺ cells (Figures 5I and 5G). In the absence of glutamine, PROX1⁺ cells, unlike the control cells, sustained both OCR and ECAR and increased glycolysis after treatment with the mitochondrial oxidative phosphorylation inhibitor trifluorocarbonylcyanide phenylhydrazide (Figures S5E and S5F). Better adaptation to glucose or glutamine starvation and oxidative phosphorylation inhibition was also observed in PROX1⁺ SW620 cells (data not shown).

In order to evaluate cell metabolism in hypoxia, we measured ECAR and OCR in cells cultured for 24 hr in 0.1% O₂. SW620 PROX1⁺ cells had higher basal level of oxidative phosphorylation in normoxia and also displayed a higher glycolysis rate in response to hypoxia (Figures S5G and S5H). Similarly, DLD1-PROX1 cells cultured in hypoxia and low glutamine displayed higher levels of glycolysis in comparison to the control cells (data not shown). Taken together, these data indicate that, whereas not favoring either oxidative phosphorylation or glycolysis, PROX1 promotes cell adaption to changes in fuel source. Such metabolic fitness of PROX1⁺ cells would confer growth advantages under unfavorable conditions that tumor cells encounter during metastasis outgrowth.

Continuous PROX1 Expression Is Necessary for Growth CRC Metastases

To evaluate the therapeutic potential of targeting PROX1, we studied whether ablation of PROX1 affects established metastases. SW480R/PROX1si cells, expressing a PROX1-targeting shRNA under the control of doxycycline, were injected intracably. Doxycycline treatment was initiated 30 days later, when both primary and metastatic lesions already developed. PROX1 depletion did not affect primary tumors, despite downregulation of PROX1 protein (Figures 6A, 6B, and S6A). However, further growth of metastases was arrested in the absence of PROX1, whereas they continued to grow in the control mice (Figures 6A, 6C, S6B, and S6C). Thus, PROX1 inhibition may be a

(F) Quantification of macrometastases size. Student's *t* test; **p* < 0.05; ****p* < 0.001. Data are presented as box and whiskers; Tukey appearance.

(G) Increased proliferation (upper row) and decreased necrosis (lower row) in SW620shCtrl liver metastasis. Staining for human vimentin (red), phosphohistone H3 (green), and DNA (blue) or chromogenic TUNEL assay (brown). The scale bars represent 50 μ m (upper) and 100 μ m (lower).

(H) Quantification of cell proliferation or apoptosis in metastases. Proliferating cells were determined by staining for phosphohistone H3 and apoptosis by TUNEL assay. Two-way ANOVA; ***p* < 0.01; ****p* < 0.001. Data are presented as mean \pm SEM.

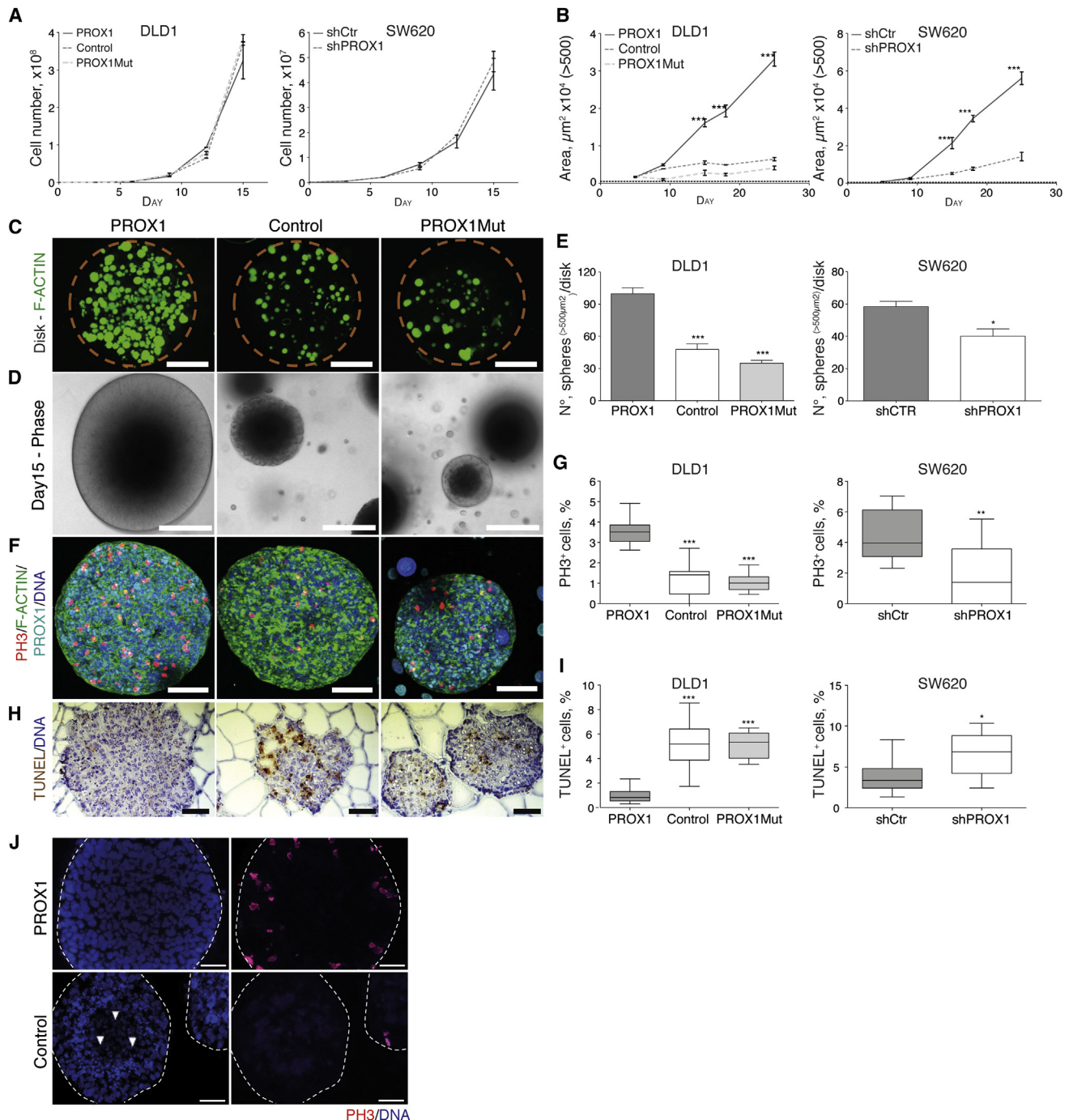


Figure 4. PROX1 Promotes Cell Growth and Survival in 3D

(A) PROX1 does not affect cell growth in 2D conditions. Growth curves of DLD1 PROX1 control or PROX1Mut and SW620 shCtr or shPROX1 cells. Two-way ANOVA; not significant (ns). Data are presented as mean \pm SEM.

(B) PROX1 promotes growth of tumor spheroids. Cells seeded in qGel were cultured in complete culture medium and spheroid size determined by phase-contrast microscopy. Threshold $>500 \mu\text{m}^2$ was used. Two-way ANOVA; *** $p < 0.001$. Data are presented as mean \pm SEM.

(C) Macroscopic view of tumor spheroids. Cells were stained for F-actin (green) 21 days after seeding. The scale bar represents 2.5 mm.

(D) Microscopic appearance of PROX1, control, or PROX1Mut spheroids at day 15. The scale bar represents 200 μm .

(E) Quantification of spheroids number at day 21. Data are presented as mean \pm SEM.

(F) PROX1 promotes proliferation of spheroids. Whole-mount staining for phosphohistone H3 (red), F-actin (green), PROX1 (light blue), and DNA (dark blue). The scale bar represents 100 μm .

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useful therapeutic option for treatment of PROX1⁺ CRC metastases.

Pharmacological Inhibition of Autophagy Targets Metastatic PROX1⁺ Cells

With few exceptions, therapeutic targeting of transcription factors is difficult (Koehler, 2010). Therefore, we sought to identify pharmacological approaches for targeting PROX1⁺ CRC cells. One of the pathways that mediate stress-induced metabolic adaptation is autophagy, a process of recycling cell components to maintain nutrient flux and energy homeostasis (White, 2012). To test whether autophagy underlies PROX1-induced survival under metabolic stress, we treated cells with chloroquine (CQ), which inhibits autophagy by preventing endosomal acidification. CQ-treated PROX1⁺ and PROX1[−] cells accumulated enlarged LC3B⁺ autophagosomes in hypoxia, demonstrating efficient autophagy inhibition (Figure S6D). CQ prevented proliferation of hypoxic PROX1⁺ cells, whereas it had little effect on PROX1⁺ or PROX1[−] cells under normoxic conditions (Figures 6D, 6E, and S5E). We also treated cells with another autophagy inhibitor, 3-methyl adenine (3-MA) (White, 2012). Similar to the CQ, 3-MA induced cell death of PROX1⁺ cells under hypoxic conditions (Figure S6F). Thus, autophagy inhibition and metabolic stress induce a synthetically lethal phenotype in PROX1⁺ cells. It also suggests that autophagy inhibition, which is now under clinical trials in metastatic CRC (<http://clinicaltrials.gov/show/NCT01006369>), may preferentially target PROX1⁺ tumor cells.

To evaluate this hypothesis, we injected mice intravenously with PROX1⁺ or PROX1[−] SW620 cells and monitored the effects of CQ on liver metastases. CQ was administered either at high (40 mg/kg; Sasaki et al., 2012) or at low (10 mg/kg) dosage, which better mimics the situation in patients (Kimura et al., 2013). The high-CQ regimen significantly reduced the size, but not the number, of liver metastases (Figures S6G and S6H). Strikingly, these macrometastases had increased necrosis, with frequently only a rim of live cells (Figures S6I and S6J). PROX1[−] cells did not form liver macrometastases at this time point, and CQ had no further effect on cell death (Figures S6G–S6I). Importantly, although low-CQ regimen did not reduce liver macrometastasis incidence or size (Figures 6F and S6K), it still strongly induced tumor necrosis and decreased cell proliferation (Figures 6G, 6H, S6L, and S6M).

Decreased angiogenesis can also lead to tumor necrosis. Therefore, we quantified tumor vessel density. Vascular density was comparable in treated versus control metastases (Figures 6H and S6N), arguing that CQ acts directly on tumor cells. Proliferating cells in CQ-treated tumors were found only at the tumor-stroma interface, characterized by abundant vasculature, showing that treated cells need oxygen- and nutrient-replete conditions to proliferate (Figures 6H, S6L, and S6N).

Intriguingly, PROX1 had little effect on orthotopically implanted primary tumors, whereas it promoted growth of metastases (Figures 2, 6A–6C, and S2). To explain this differential response, we analyzed proliferation and apoptosis in metastases versus primary tumors. In two different models, PROX1⁺ and PROX1[−] primary tumors proliferated at low and comparable rates and had low levels apoptosis (Figures 6I, 6J, and S6O; data not shown). In contrast, proliferation was high in PROX1⁺ metastases, whereas apoptosis was induced in PROX1[−] metastases (Figures 6J and S6O). Because only PROX1⁺ cells can productively use autophagy (Figure 6D), we compared responses to autophagy inhibition of primary tumors and metastases. Treatment with CQ did not affect primary tumor proliferation (Figures 6K, 6L, and S6P) or metastasis incidence (Figure S6Q). However, CQ significantly suppressed proliferation of PROX1⁺ metastases (Figures 6K and 6L). Furthermore, although CQ treatment did induce some necrosis in primary tumors, necrosis levels were significantly higher in PROX1⁺ metastases (Figure 6M). Importantly, the treatment had no effect on vascular density (Figure S6R).

To better understand why PROX1⁺ metastases respond better than primary tumors to autophagy inhibition, we next compared autophagy in primary versus metastatic lesions by quantifying differences in LC3B levels between the control and CQ-treated lesions. Such autophagic flux is a more accurate measure of autophagy than the steady-state level of processed LC3B (Mizushima et al., 2010). Autophagic flux was higher in metastases, regardless of the levels of PROX1 (Figures 6N–6Q). Our data thus show that CQ efficiently targets PROX1⁺ cells in macrometastases. They also indicate that the metastatic CRC cells have higher levels of autophagy, which points to increased metabolic stress in the sites of metastases. Although the mechanisms remain to be elucidated, these results show that PROX1⁺ metastases may respond to autophagy inhibition in patients.

BCL2L15 Is a Direct Target of PROX1, which Suppresses Tumor Growth

To study the transcriptional network regulated by PROX1, we carried out chromatin immunoprecipitation sequencing (ChIP-seq) and transcriptome analysis after small interfering RNA (siRNA)-mediated knockdown of PROX1 in SW480R cells (Figure S7A). The analyses identified 23,450 PROX1-binding sites (false discovery rate [FDR] < 0.001) and 444 PROX1-dependent genes (FDR < 0.05 and fold change [FC] > 1.5). To find how PROX1 DNA binding correlates with changes in gene expression, we mapped these genes to nearby ChIP-seq-binding regions. Unaffected genes (mean expression value >8 and FC < 1.5) were used as reference. We observed that PROX1 preferentially binds in the vicinity of genes induced by PROX1 depletion (Figure S7B). Thus, as found in other cell types (e.g., Steffensen et al., 2004), PROX1 acts as a transcriptional repressor.

(G) Quantification of proliferation in tumor spheroids. Data are presented as box and whiskers; Tukey appearance.

(H) Decreased apoptosis in PROX1-expressing spheroids. Staining for TUNEL assay (brown) and DNA (light blue). The scale bar represents 100 μ m.

(I) Quantification of apoptosis, shown as percentage TUNEL⁺ cells in sections. DLD1, one-way ANOVA; ***p < 0.001. SW620, Student's t test; ***p < 0.001. *p < 0.05; **p < 0.01. Data are presented as box and whiskers; Tukey appearance.

(J) Necrotic core is present in control but PROX1-expressing spheroids. Staining for PH3 (red) and DNA (blue). White arrowheads, necrotic areas. The scale bar represents 50 μ m.

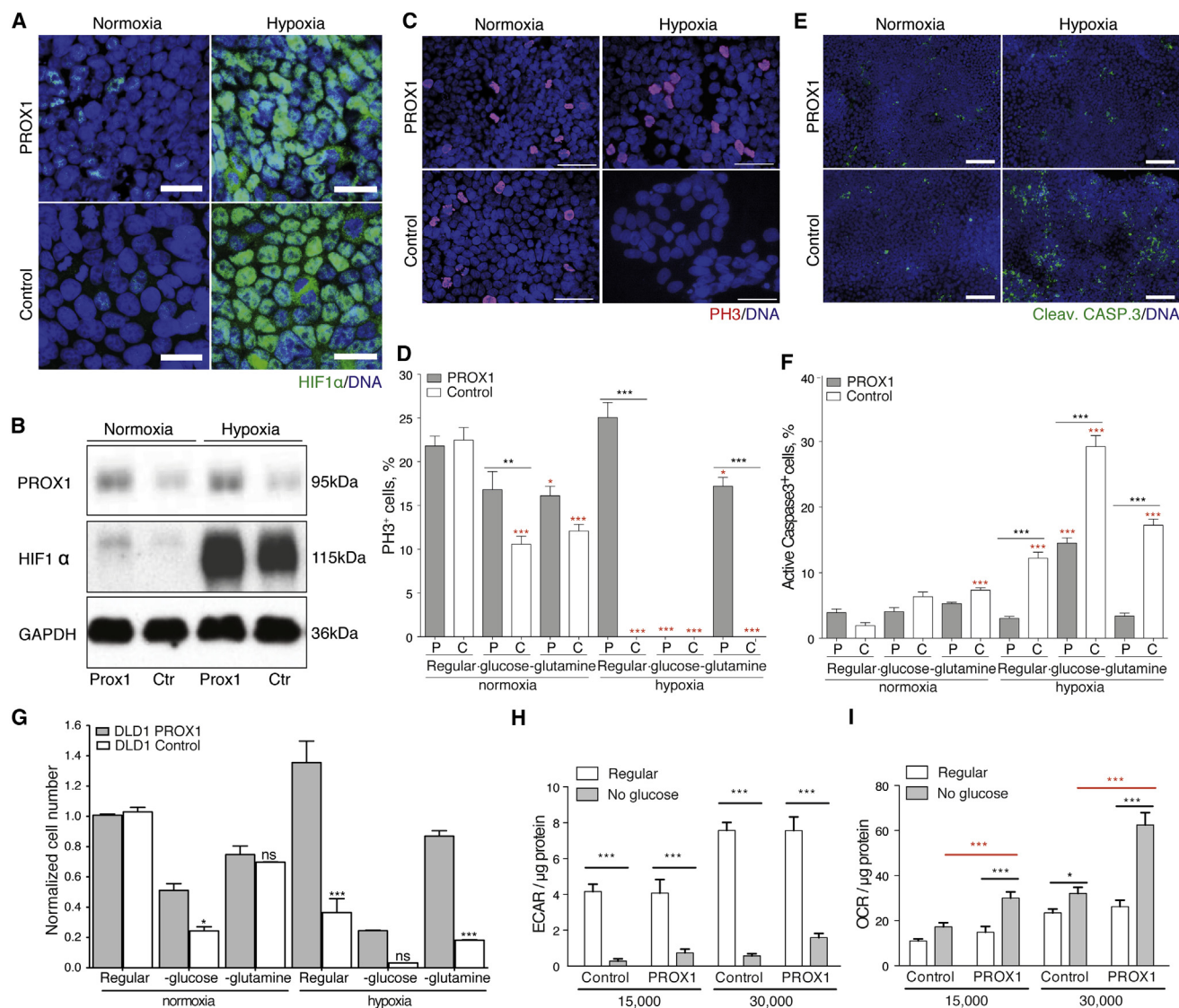


Figure 5. PROX1 Promotes Metabolic Adaptation In Vitro

(A) HIF1 α is stabilized by hypoxia both in DLD1-PROX1 or control cells. Staining for HIF1 α (green) and DNA (blue). The scale bar represents 50 μ m.

(B) HIF1 α levels in PROX1⁺ or PROX1⁻ cells after 12 hr in hypoxia. Western blotting for the indicated proteins.

(C) PROX1⁺ cells proliferate in hypoxia. Cells were cultured in normoxia or 0.1% O₂ for 72 hr. Staining for PH3 (red) and DNA (blue). The scale bar represents 50 μ m.

(D) PROX1⁺ cells proliferate under metabolic stress. Cells were cultured in the indicated conditions for 72 hr and stained for phosphohistone H3 and DNA. Data are presented as mean \pm SEM.

(E) PROX1 reduces cell apoptosis under metabolic stress conditions. Staining for cleaved caspase-3 (green) and DNA (blue) after 72 hr. The scale bar represents 200 μ m.

(F) Quantification of apoptosis for data shown in (E). One-way ANOVA; **p < 0.01; ***p < 0.001. Black *, PROX1 versus control. Two-way ANOVA; *p < 0.05; ***p < 0.001. Red *, treatment versus complete medium in normoxia. Data are presented as mean \pm SEM.

(G) Quantification of DLD1 cell number after 3 days incubation in the indicated conditions. *p < 0.05; ***p < 0.001. Cell number was determined by CyQuant assay. Data are presented as mean \pm SEM.

(H) Glucose deprivation decreases lactate production in both DLD1-PROX1 and control cells. ECAR was measured in cells cultured at two different densities. Data are presented as mean \pm SEM.

(I) PROX1⁺, but not control, cells increase oxidative phosphorylation in the absence of glucose. OCR was measured in the same samples as for (H). Data are presented as mean \pm SEM. Two-way ANOVA; *p < 0.05; **p < 0.005; ***p < 0.001; red *, PROX1 versus control in indicated condition; black *, significant change of the same cell type in different conditions.

As PROX1 promoted survival of both DLD1 and SW480R cells, we reasoned that important direct targets should be repressed in both cell lines. To identify such targets, we combined ChIP-seq data with gene expression profiling results in two cell types. We identified 40 genes, repressed by PROX1 and carrying PROX1-binding sites (FDR < 0.05; [Tables S1, S2, and S3](#)), out of which seven genes (*ABAT*, *BCL2L15*, *CAV1*, *IQGAP2*, *SEMA3C*, *SLC7A7*, and *TNFRSF9*) were repressed by PROX1 more than 2-fold ([Figures 7A, S7C, and S7D](#)). For most of these genes, the direct connection to CRC progression remains to be investigated. However, BCL2 family member, BCL2L15, acts as a conditional pro-apoptotic gene in CRC cells in vitro ([Dempsey et al., 2005](#)) and *Cav1*^{-/-} mice have more aggressive lesions in a genetic model of intestinal cancer ([Friedrich et al., 2013](#)).

We tested next whether metabolic stress conditions affected the expression of BCL2L15 and CAV1. Hypoxia induced expression of BCL2L15 and CAV1 in PROX1⁻, but not PROX1⁺ cells, suggesting that they act as PROX1 effectors in cells under metabolic stress ([Figures 7B and 7C](#)). At the same time, PROX1⁺ and PROX1⁻ cell response to hypoxia was comparable, as judged by expression levels of the HIF1 α target VEGF-A ([Figure 7D](#)). We overexpressed BCL2L15, CAV1, or another PROX1 target TNFRSF9 in PROX1⁺ CRC cells ([Figure S7E](#)) and tested growth and survival of these cells under normoxia and hypoxia. BCL2L15 and, to a lesser extent, CAV1 reduced proliferation and increased apoptosis of PROX1⁺ cells under hypoxia ([Figures 7E, 7F, and S7F](#)). In contrast, overexpression of TNFRSF9 had no effect ([Figure S7F](#)). Furthermore, BCL2L15 overexpression in PROX1⁺ CRC cells drastically reduced the number and size of metastases after intravenous (i.v.) injections ([Figures 7G–7J](#)). To test the effects of BCL2L15 inactivation, we used the AKP intestinal organoid model, in which Bcl2l15 is expressed in Prox1-negative cells and is further induced after Prox1 depletion ([Figures S7G and S7H](#)). We found that Bcl2l15 depletion enhanced clonogenic growth of intestinal organoids in vitro ([Figures 7K and S7I](#)), as well as proliferation and growth of organoid-derived tumors in vivo ([Figures 7L–7N and S7J](#)), without affecting cell survival ([Figure 7O](#)). As expected, Prox1 depletion reduced colony formation and tumor growth under the same conditions ([Figures 7K–7O, S7I, and S7J](#)).

To further analyze the significance of BCL2L15 and CAV1 regulation by PROX1 in human CRC in vivo, we studied BCL2L15 and CAV1 protein localization in human CRC. BCL2L15 and PROX1 displayed a mutually exclusive localization pattern both in metastases and primary tumors ([Figures 7P and S7K](#); data not shown). In contrast, CAV1 was generally low in CRC cells independently of PROX1 status (data not shown). These results suggest that BCL2L15 suppression is a more-specific marker for PROX1 activity. Moreover, luciferase reporter assays showed that PROX1 directly repressed the activity of the 3' PROX1-binding region in the BCL2L15 locus ([Figures S7L and S7M](#)). BCL2L15 mRNA levels were also strongly reduced in CRC tumors (n = 230; p = 2.11 $\times 10^{-8}$; [Figure 7Q](#)) and showed a significant negative correlation with PROX1 ([Figure 7Q](#); p = 1.8 $\times 10^{-4}$). In summary, our genome-wide analyses identified a number of potential direct PROX1 targets in CRC ([Table S1](#)), whose roles still need to be investigated. Here, we show that BCL2L15 is an important, even if likely not the only, effector of

PROX1 in CRC, whose suppression in part recapitulates the effects of PROX1.

DISCUSSION

We report that the colon-cancer-specific Wnt target PROX1 is highly expressed in colon cancer metastases, where it is present in a subset of cancer stem/progenitor cells. We also show that, in advanced CRC, PROX1 sustains growth of macrometastases through the evasion of apoptosis and metabolic adaptation. Previous studies investigated the mechanisms of local invasion in primary CRC tumors. They established important roles for Wnt and Notch signaling, EMT, and the tumor-stroma interactions (e.g., [Dolznic et al., 2011](#); [Sonoshita et al., 2011](#); [Spaderna et al., 2008](#); [Wolf et al., 2012](#)). However, mechanisms governing outgrowth of metastases are not well understood. Yet metastatic CRC is a major clinical challenge, with limited patient survival and few therapeutic options available, especially for patients with activating KRAS mutations, which confer resistance to anti-EGFR therapy ([Cunningham et al., 2010](#); [Van Emburgh et al., 2014](#)).

In this study, we investigated the role of the transcription factor PROX1 in the development of CRC metastases. Expression of PROX1 in primary CRC tumors is not an independent prognostic factor ([Skog et al., 2011](#)). However, we found that PROX1 levels are generally increased in metastases and a proportion of PROX1⁻ primary tumors give rise to PROX1⁺ metastases. Large expression data sets from metastatic lesions will clarify whether high levels of expression of PROX1 in a metastasis correlates with survival after relapse. Nevertheless, already now our data suggest that, in advanced CRC, PROX1 may be important in the context of metastatic disease.

Analysis of PROX1 expression patterns in human metastases and mouse models demonstrated that PROX1 is expressed only upon transformation and it may delineate a distinct subpopulation of cancer stem/progenitor cells. Furthermore, in vitro analyses of the intestinal organoid model demonstrated that, while Prox1 does not directly control the expression of stem/progenitor-associated genes or general Wnt pathway activity, it is essential to maintain clonogenic growth. Our data are in agreement with the results of Wiener et al., who show that Prox1 is essential for the expansion of the stem cell pool in transformed but not in normal intestinal stem cells ([Wiener et al., 2014](#)). Through the analysis of mouse models of CRC metastasis, we found that PROX1 is dispensable for growth of primary tumors, cell survival in the bloodstream, or extravasation; however, it promotes outgrowth of metastases. At least in part, this property of PROX1 relies on its ability to override cell death imposed by metabolic stress, such as hypoxia or low-nutrient environment. Taken together, our data suggest that, in CRC metastases, there exists a subpopulation of PROX1⁺ stem/progenitor cells, which are highly resistant to metabolic insults and can thus fuel metastatic growth. These data identify PROX1 as an important player in CRC stem/progenitor cells maintenance and function.

To investigate the mechanisms of PROX1 action in CRC, we carried out PROX1 genome location analysis and identified genes regulated by PROX1. As expected, a significant number of direct PROX1 target genes were identified ([Table S1](#)), and

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we began investigating their individual contribution. Here, we show that a pro-apoptotic member of the BCL2 family, BCL2L15 (Dempsey et al., 2005), is induced in hypoxic PROX1[−], but not PROX1⁺, cancer cells and that BCL2L15 can in part recapitulate the phenotype of PROX1 deficiency, such as increased clonogenic growth of tumor organoids or tumor development in vivo. These results indicate that apoptosis evasion under metabolic stress conditions is important for the prosurvival function of PROX1⁺ in metastases. Most likely, the role of other potentially interesting targets and their combinatorial interactions will need to be addressed in the future. Of interest, Annexin A1, identified as a PROX1 effector by Wiener et al. (2014) is another direct target of PROX1 in our data set (Table S1).

With few exceptions, transcription factors are considered undruggable. To overcome this limitation, we searched for pharmacological approaches to target PROX1⁺ tumor cells. We found that inhibition of autophagy, a catabolic process providing an alternative energy source, eliminates the growth advantage of PROX1⁺ CRC cells and decreases metastatic burden. Of interest, following CQ treatment, viable tumor cells are located in the vicinity of blood vessels and they remain accessible for cytotoxic drugs. Thus, a combination of targeted anti-PROX1 therapy with antiproliferative/cytotoxic 5-fluorouracil-based chemotherapy might be pursued as a potential treatment option for CRC patients with PROX1⁺ metastatic disease.

We propose the following working model of PROX1's role in metastasis: high Wnt signaling induces PROX1 expression in CRC cells and PROX1⁺ cancer stem/progenitor cells become

resistant to metabolic stress, in part through the repression of BCL2L15. Because PROX1 is produced only in CRC, but not normal stem cells, and metabolic stress is high in metastases, these data provide one of the mechanisms of how oncogenic Wnt signaling induces cancer cell adaptation and promotes metastatic cell outgrowth.

A related intriguing question is whether increased fitness of PROX1⁺ tumor cells reflects the role of PROX1 in normal tissues. The highest levels of Prox1 are found in lens fiber cells (Oliver et al., 1993). The lens is an avascular organ, located in a physiologically hypoxic and glucose-poor environment (Mathias et al., 1997; Trayhurn and Van Heyningen, 1972), and it is not fully understood how the lens maintains homeostasis in such conditions. Similarly, PROX1⁺ lymphatic vessels, unlike blood vessels, do not need basement membrane or pericytes to survive, grow in relatively hypoxic environment, and do not regress after removal of proliferative stimuli (Baluk et al., 2005). Thus, it seems plausible that PROX1 enhances adaptation of normal cells to unfavorable metabolic conditions.

An important observation of this study is the differential role of PROX1 in metastases versus primary tumors in advanced colorectal cancer. In our models, all of which carry mutations in oncogenic and tumor suppressor genes APC, KRAS, and TP53, PROX1 suppression decreased metastasis size and proliferation, whereas having little effect on the size or proliferation of primary tumors. Whereas the mechanisms are yet unknown, we propose that PROX1 is needed to provide a survival advantage to metabolically stressed cells. This is supported by the fact that CQ treatment is more effective in reducing growth of

Figure 6. Therapeutic Potential of Targeting PROX1

- (A) PROX1 depletion in established metastases prevents further growth. SW480R/PROX1si cells, implanted intracably into NOD-SCID; IL-2R $\gamma^{-/-}$ mice, were detected by in vivo bioluminescence imaging. Insert: macroscopic liver metastases in control SW480R/PROX1si mouse. The scale bar represents 750 μ m. Two-way ANOVA; ***p < 0.001. Data are presented as mean \pm SEM.
- (B) PROX1 suppression does not affect weight of primary tumors. Data are presented as box and whiskers; Tukey appearance.
- (C) PROX1 suppression prevents further growth of metastases. ***p < 0.001. Data are presented as box and whiskers; Tukey appearance.
- (D) Inhibition of autophagy prevents PROX1-dependent adaptation to hypoxia. CQ, chloroquine. SW620 shCtr or shPROX1 cells were cultured for 72 hr in normoxic or hypoxic conditions in the presence or absence of 5 μ M CQ. Staining for phosphohistone H3 (red), GLUT1 (green), and DNA (blue). The scale bar represents 50 μ m.
- (E) Quantification of cell proliferation. *p < 0.05; ***p < 0.001. Data are presented as mean \pm SEM.
- (F) Low-dose CQ does not affect metastases size. Quantification of metastases area in mice injected i.v. with tumor cells and treated with 10 mg/g CQ or PBS from day 7 post-implantation. Both necrotic and live tumor areas of metastases >100 μ m² were quantified. Data are presented as box and whiskers; Tukey appearance.
- (G) CQ treatment induces necrosis in metastases. Quantification of necrotic areas in liver metastases in mice treated with 10 mg/g CQ or PBS. Necrosis was evaluated at tumor-stromal interface, metastasis center, and in the entire lesion. **p < 0.01; ***p < 0.001. Data are presented as mean \pm SEM.
- (H) Low-dose CQ does not affect blood-vessel density. Left column: staining for endothelial marker endomucin (green), phosphohistone H3 (light blue), and DNA (blue). Yellow scattered line: highly vascularized metastatic area close to the liver stroma. White scattered line: the necrotic area. The scale bar represents 200 μ m. Right column: staining for PROX1 (red), blood vessel marker endomucin (green), and DNA (blue). The scale bar represents 100 μ m.
- (I) CQ treatment reduces proliferation of PROX1⁺ metastases but has little effect on primary tumors. Staining for PH3 (light blue), PROX1 (red), and DNA (blue). White scattered lines, tumor area; white asterisk, areas of necrosis; yellow arrows, PH3⁺ cells. The scale bar represents 100 μ m.
- (J) Quantification of proliferation in orthotopic SW620 shCtr or shPROX1 primary tumors or metastases. Two-way ANOVA; ***p < 0.001. Data are presented as mean \pm SEM.
- (K) CQ treatment reduces proliferation of PROX1⁺ metastases. Staining for EdU (red), PROX1 (light blue), pimonidazole (green), and DNA (blue) of orthotopic SW620 shCtr or shPROX1 primary tumors or metastases following treatment with PBS or CQ. The scale bar represents 200 μ m.
- (L) Quantification of proliferation as determined by EdU incorporation. Data are presented as mean \pm SEM.
- (M) CQ treatment induces necrosis in PROX1⁺ metastases. Data are presented as mean \pm SEM.
- (N) Higher autophagic flux in metastases as compared to primary tumors. Staining for LC3B (red) and DNA (blue). Hypoxic areas are identified by staining for pimonidazole (green). The scale bar represents 25 μ m.
- (O) Quantification of LC3B⁺ cancer cells in primary tumors and metastases. Data are presented as mean \pm SEM.
- (P) Quantification of LC3B⁺ foci in individual cells. Two-way ANOVA; *p < 0.05; **p < 0.01. Data are presented as mean \pm SEM.
- (Q) Examples of images used for quantification in (O) and (P). Staining for LC3B (red) and DNA (blue). The scale bar represents 2 μ m.

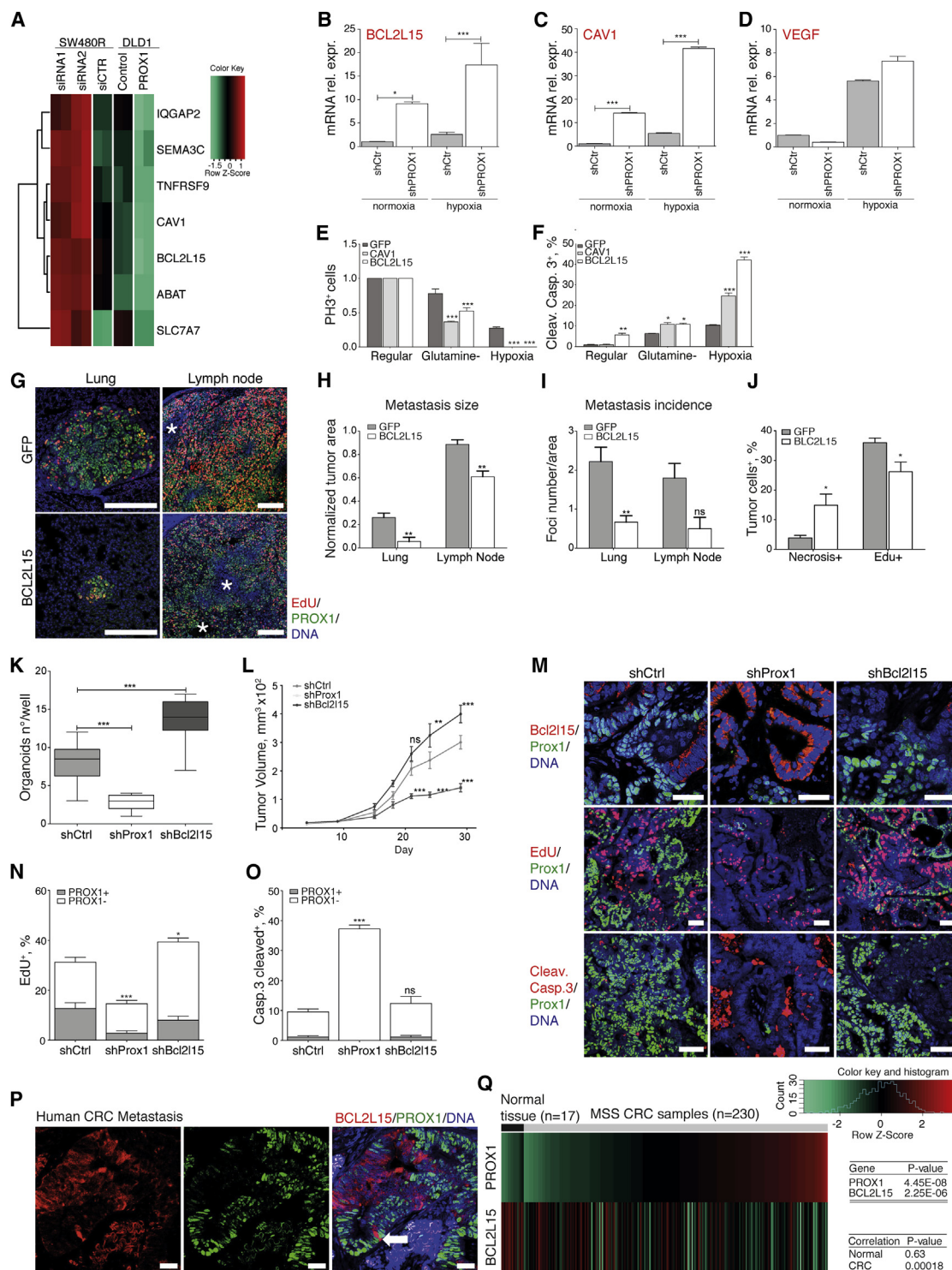


Figure 7. BCL2L15 Is a Direct PROX1 Effector Gene

(A) Heatmaps of selected PROX1 targets in two cell lines.
(B–D) BCL2L15 and CAV1 are induced in hypoxia in the absence of PROX1. Quantitative PCR analysis of the indicated transcripts. Cells were cultured in normoxia or hypoxia for 24 hr. Data are presented as mean \pm SEM.
(E and F) BCL2L15 and CAV1 suppress cell proliferation and induce apoptosis in hypoxia. Cells were cultured for 72 hr in normoxia or 0.1% O₂. Proliferating or apoptotic cells were identified by staining for phosphohistone H3 or activated caspase-3. Two-way ANOVA; *p < 0.05; **p < 0.01; ***p < 0.001. Data are presented as mean \pm SEM.

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PROX1⁺ metastasis versus primary tumors and that metastases have higher levels of autophagy. Interestingly, whereas generally PROX1 increases metastases size, PROX1⁺ and PROX1⁻ metastases have a comparable size in skeletal muscle (Figure 3E). These data indicate that metabolically active tissue such as skeletal muscle can complement the deficiency in PROX1. They also highlight important differences in metabolism of metastases versus primary tumors and further underscore a need to better understand and exploit such differences.

Previous studies documented a critical role of stromal inflammation, extracellular matrix, and chemokine/growth factor signaling for the successful outgrowth of cancer cells (Chaffer and Weinberg, 2011; Bhowmick, 2012; Zeelenberg et al., 2003). Our work reveals a cancer-type-specific mechanism of metastasis progression by demonstrating that the CRC-specific Wnt target gene PROX1 increases metabolic fitness of metastatic cancer stem cells. Importantly, all human and mouse models used in the study carry an activating mutation in *KRAS*, which underlies intrinsic or acquired resistance to targeted anti-EGFR therapies (Van Emburgh et al., 2014). PROX1 deficiency can therefore override advantages gained through the activation of *KRAS* signaling in Wnt^{high} cancer cells. Thus, targeting of PROX1 and PROX1-dependent pathways should be further explored for development of potential treatment approaches of PROX1⁺ *KRAS* mutant, anti-EGFR-resistant CRC metastases.

EXPERIMENTAL PROCEDURES

Human Tumor Analyses

Use of human CRC samples was approved by the Canton of Vaud's ethics committee. See [Supplemental Experimental Procedures](#) for staining and tumor sample information.

Bioinformatics Analyses

The correlation analysis of PROX1 expression with cell differentiation, proliferation, and stem cell markers was based on GSE41258 data set (Sheffer et al., 2009). We used GSE33112 data set (de Sousa E Melo et al., 2011) to analyze PROX1 levels in Wnt^{high} and Wnt^{low} CSCs. See [Supplemental Experimental Procedures](#) for the detailed description. PROX1 and BCL2L15 expression was analyzed in 230 microsatellite stable colon tumors and 17 normal colon tissues from The Cancer Genome Atlas (TCGA) data set COAD UNC_illumina-GA_RNASeqV2 (TCGA project: <http://cancergenome.nih.gov/>).

Cell Lines and Culture Conditions

SW620, SW480, and DLD1 (CCL-227, CCL-228, and CCL-221; ATCC) cells were used for generation of cell lines and spheroids as described in [Supplemental Experimental Procedures](#). ECAR and OCR were measured in cells cultured for 24 hr prior to analysis according to the manufacturer's protocols (SeahorseBio), and all data were normalized to the total protein content.

Animal Studies

Experiments were approved by the Animal Ethics Committee of the Canton of Vaud or by the UK Home Office regulations. The complete list of mouse lines, genotyping, and other procedures are provided in [Supplemental Experimental Procedures](#).

Staining Procedures

Staining procedures and list of antibodies are provided in [Supplemental Experimental Procedures](#). Metastases were quantified using staining for human vimentin or KRT20. We detected cell proliferation for phosphohistone H3 (Cell Signaling Technology) and apoptosis by staining for cleaved caspase-3 (CST) or by TUNEL assay (R&D Systems). We used Axiovision Imager.Z1 or Zeiss LSM 510 META scanning confocal microscope for image acquisition and Bitplane IMARIS Suite 6.3.1 and Adobe Photoshop CS6 for image analyses.

RNA Isolation, Quantitative PCR, Microarray Analyses, and Western Blotting

We used RNeasy columns (QIAGEN) for RNA isolation and Agilent 2100 Bio-analyzer (Agilent) for RNA quality control. For transcriptome analyses, we used RNA from DLD1, DLD1-PROX1, or SW480R cells, transfected with 10 nM PROX1 siRNAs and AllStars control siRNAs (QIAGEN). Affymetrix Human Gene 1.0 ST arrays (Affymetrix) were used for gene-expression profiling.

See [Supplemental Experimental Procedures](#) for list of siRNAs and primers. We performed RT-PCR analyses on StepOnePlus or 7500-Fast real-time PCR Systems (Applied Biosystems) with SYBR Green PCR Master Mix (Applied Biosystems).

For western blotting, proteins were resolved by SDS-PAGE, transferred onto Immobilon-P, and detected with horseradish-peroxidase-conjugated secondary antibodies (Dako) and enhanced chemiluminescence (Thermo Fisher Scientific).

PROX1 ChIP-Seq and Reporter Analyses

Pools of three independent ChIP experiments and input DNA were used for sequencing (see [Supplemental Experimental Procedures](#)).

ChIP-seq data, generation of BCL2L15 luciferase reporter constructs, and their analyses are described in [Supplemental Experimental Procedures](#).

(G) BCL2L15 limits growth of metastases. GFP- or BCL2L15-expressing SW620 cells were injected i.v., and metastases were analyzed 30 days later. Staining for PROX1 (green), EdU (red), and DNA (blue). The scale bar represents 100 μ m.

(H) Quantification of metastasis size for data shown in (F). Data are presented as mean \pm SEM.

(I) Metastasis incidence. Data are presented as mean \pm SEM.

(J) BCL2L15 promotes tumor necrosis and reduces proliferation in metastatic PROX1⁺ cells. Two-way ANOVA; * $p < 0.05$; ** $p < 0.01$. Data are presented as mean \pm SEM.

(K) Depletion of Bcl2l15 increases clonogenicity of intestinal *Apc^{fl/fl};Kras^{G12D};Tp53^{fl/fl};villin-CreERT2* (AKP) organoids. Colonies were quantified at day 15, following seeding of 3,000 single cells per well, transduced with a nontargeting shRNA (shCtrl) or shProx1. One-way ANOVA; *** $p < 0.001$. Data are presented as box and whiskers; Tukey appearance.

(L) Bcl2l15 depletion promotes tumor growth. shBcl2l15, control nontargeting shRNA (shCtrl), or shProx1-transduced organoid were implanted subcutaneously into NOD-SCID $\gamma^{-/-}$ mice. Two-way ANOVA; ** $p < 0.01$; *** $p < 0.001$. Data are presented as mean \pm SEM.

(M) Target protein expression, proliferation, and apoptosis in organoid-derived tumors. Staining for Prox1 (green), Bcl2l15, EdU or cleaved caspase-3 (red), and DNA (blue). The scale bar represents 25 μ m.

(N) Quantification of cell proliferation in organoid tumors. Data are presented as mean \pm SEM.

(O) Quantification of apoptosis in organoid tumors. One-way ANOVA; * $p < 0.05$; *** $p < 0.001$. Data are presented as mean \pm SEM.

(P) Mutually exclusive localization of PROX1 and BCL2L15 in human CRC metastases. Staining for PROX1 (green), BCL2L15 (red), and DNA (blue). The scale bar represents 25 μ m.

(Q) BCL2L15 mRNA is reduced in colon tumors and anticorrelates with PROX1 mRNA levels. Heatmap of PROX1 and BCL2L15 mRNA levels in CRC tumors and normal colon samples.

Statistical Analyses

We used Prism 5.0 for laboratory data analysis. p value ≤ 0.05 was considered to be statistically significant. Full description of statistical analyses is provided in [Supplemental Experimental Procedures](#).

ACCESSION NUMBERS

The ArrayExpress accession numbers for the DLD1 or SW480R \pm PROX1 microarray data reported in this paper are E-MTAB-2020 and E-MTAB-2021. The Gene Expression Omnibus submission accession number for the PROX1 ChIP-seq analysis performed on SW480R cells reported in this paper is GSE60390. The Cis-Regulatory Element Annotation System output relative to those data is accessible at http://bcf.isb-sib.ch/data/ragusa_et_al/PROX1vsinput_hg18.bed and http://bcf.isb-sib.ch/data/ragusa_et_al/.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and four tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.08.041>.

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